

X-chromosome inactivation mosaicism in the three germ layers and the germ line of the mouse embryo

By A. McMAHON¹, M. FOSTEN² AND M. MONK^{2, 3}

From the MRC Mammalian Development Unit, University College London

SUMMARY

Electrophoretic variant forms of the X-linked enzyme phosphoglycerate kinase (PGK-1, E.C.2.7.2.3) have been used to examine X-chromosome mosaicism in tissues from 12½-day *post coitum* heterozygous female mouse embryos. Samples of yolk-sac endoderm, neural ectoderm, heart (mesoderm), liver (endoderm) and germ cells were analysed from each embryo. In all tissues except yolk-sac endoderm, both PGK-1 isozymes were expressed. The extent of covariance among tissues with respect to the PGK-1 isozyme contribution is consistent with all tissues being derived from the same pool of cells after X-inactivation. The covariance among tissues gives an estimate of the size of this pool (47 cells) and places the earliest time of X-inactivation in epiblast cells between 4½ and 5½ days *post coitum*. From the independent variance among tissues within an individual, the average primordial precursor pool size for the three germ layers and the germ line itself was estimated as 193 cells.

INTRODUCTION

Genetic mosaicism in mammals has been used to examine events which take place in embryonic development (see reviews by Nesbitt & Gartler, 1971; Mintz, 1974; Gardner, 1978; West, 1978). The basic approach in these studies has been to estimate the proportions, and wherever possible, the spatial relationship, of two genetically distinct cell types in tissue samples. Genetic mosaicism may be produced experimentally in chimaeras formed by aggregation of genetically distinct embryos (Tarkowski, 1961; Mintz, 1962) or by injection of genetically distinct cells into blastocysts (Gardner, 1968). Functional genetic mosaicism also occurs naturally following X-chromosome inactivation (Lyon, 1961) in female mammals heterozygous for X-linked genes.

X-chromosome inactivation occurs early in embryonic development; it is cell autonomous and irreversible (except in the germ line), giving rise to somatic clones with either the maternal or the paternal X chromosome active. In X-inactivation mosaics all cells are genetically identical except at heterozygous

¹ *Author's current address:* Division of Biology, California Institute of Technology, Pasadena, C.A. 91125, U.S.A.

² *Authors' address:* MRC Mammalian Development Unit, Wolfson House (University College London), 4 Stephenson Way, London NW1 2HE, U.K.

³ To whom reprint requests should be sent.

X-chromosome loci, and hence cell selection favouring one cell over another is less likely than in chimaeras (see McLaren, 1976a).

If we assume that the allocations of cells to different lineages and tissues are random sampling events, then statistical analysis of the compositions of differentiated mosaic tissues can yield information on primordial precursor cell pool sizes and cell lineage relationships. The statistics rely on the analysis of variation in mosaic composition among mosaic individuals and the variation and correlation of mosaic composition among different tissues within mosaic individuals. Using this approach Nesbitt (1971) demonstrated correlations in mosaic composition in melanocytes of ectodermal origin and several tissues of predominantly mesodermal origin. She scored late replication of the elongated translocation X chromosome in tissues of female mice heterozygous for Cattanach's translocation (Cattanach, 1961). Good correlation was found between all pairs of tissues from a particular female, suggesting that the tissues examined were derived from the same pool of X-inactivated cells. From the covariance in mosaic composition among tissues, the size of this pool was estimated to be at least 13 and probably about 21 cells. As tissues of both mesodermal and ectodermal origin shared the same pool Nesbitt concluded that this was the number of cells at the time of X-inactivation. The sizes of precursor pools for the different tissues calculated from the independent variance among tissues was shown to be not less than 20 to 30 cells.

It is necessary to assume in all studies measuring mosaic composition that cell selection does not favour one of the cell types. Nesbitt's (1971) analysis used tissues of 5- to 7-day-old animals and required an appreciable period of cell culture during which particular cell types may divide more rapidly than others. Disteché, Eicher & Latt (1979) have demonstrated that cell selection may occur in female mice heterozygous for Cattanach's translocation. Cell selection has also been observed in some populations of females heterozygous for X-linked enzyme deficiency (Nyhan *et al.* 1970; Fialkow, 1973) but it is less likely in females heterozygous for electrophoretic variant enzyme forms (Fialkow, 1973).

In this work we have quantitated electrophoretic variant forms of the X-linked enzyme phosphoglycerate kinase (PGK-1; E.C.2.7.2.3) in 12½-day *post coitum* heterozygous female mouse embryos. Several tissues derived from the epiblast and representative of the three definitive germ layers, and the germline were studied. The use of earlier foetal stages, and direct analysis of electrophoretic variant enzyme forms, make it unlikely that cell selection affects the mosaic composition of tissues. Also the quantitation of PGK-1 isozymes in extracts of large segments of embryonic tissue (neural ectoderm) or whole embryonic organs (heart and liver) will obviate possible variability expected in smaller samples where patchiness will arise from coherent clonal growth. Statistical analysis of the data was undertaken to investigate lineage relationships among tissues, the size of the embryo precursor pool, and the number of cells sampled for the tissue primordia.

MATERIALS AND METHODS

Mice

The PGK-1A mouse stock (kindly provided by Dr John West) was derived from a feral population of *Mus musculus* (Nielsen & Chapman, 1977) and made congenic with the inbred C3H strain (West & Chapman, 1978). Random-bred MF1 mice (OLAC) were used as a source of the *Pgk-1^b* allele in matings to produce heterozygous female embryos.

Dissections and sample collections

Pregnant females were killed 12½ days following copulation. Embryos were dissected into PB1 (Whittingham & Wales, 1969) containing 0.4 % polyvinylpyrrolidone (PVP) instead of albumin. Embryonic gonads were sexed by their characteristic morphology (developing testes show cords).

From the female embryos, yolk-sac endoderm (primary endoderm lineage) yolk-sac mesoderm, neural ectoderm, heart (mesoderm) and liver (endoderm) were collected (see Fig. 1). The yolk sac was dissected free of the embryo, and the endoderm and mesoderm layers loosened by digestion in 0.5 % trypsin (Sigma) and 2.5 % pancreatin (BDH) in calcium- and magnesium-free phosphate-buffered saline (PBS) for 1 h at 4°C (Levak-Švajger, Švajger & Škreb, 1969). Further enzyme digestion was prevented by placing the yolk sac in PB1 containing 10 % foetal calf serum (FCS). After 30 min at 4°C the endodermal and mesodermal layers were separated using watchmakers' forceps.

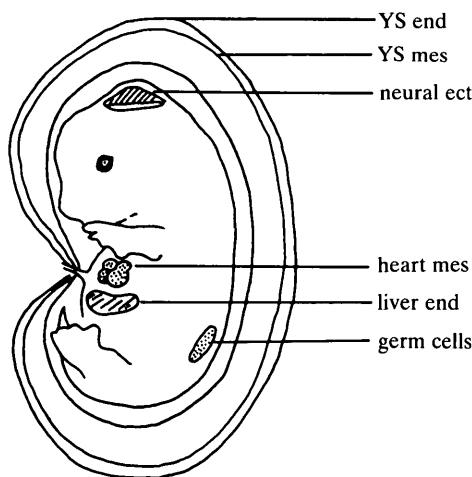


Fig. 1. Tissue sample collections from 12½-day *post coitum* female embryos heterozygous for *Pgk-1*. The yolk sac was separated into yolk-sac endoderm (YS end) and yolk-sac mesoderm (YS mes). From the embryo proper, neural ectoderm (neural ect), heart (heart mes) and liver (liver end), were collected as tissues representative of the ectodermal, mesodermal and endodermal lineages respectively. Germ cells were collected from the foetal gonad.

All samples were washed three times in PBS and stored frozen at -70°C .

Gonads were also dissected from female embryos, washed three times in PBS and the germ cells isolated by slitting the gonads with electrolytically sharpened tungsten needles and squeezing. Germ cells were then collected in a pulled Pasteur pipette in approximately $1\ \mu\text{l}$ of PBS. Germ cells could be distinguished from the principal somatic cell contamination (blood cells) by their large size and lack of pigmentation.

Evaluation of PGK-1 isozyme expression in tissue samples

All tissue samples, excluding the germ-cell collections were sonicated briefly to lyse the cells, and centrifuged. The germ-cell samples were freeze-thawed three times in liquid nitrogen and centrifuged. Samples of supernatant were analysed by Cellogel electrophoresis using the techniques of Bücher *et al.*, 1980 (see also McMahon, Fosten & Monk, 1981, and Legend to Table 1). We are indebted to Prof T. Bücher and I. Linke for instruction in these techniques.

RESULTS

PGK-1 isozyme expression was examined in six tissues from eight $12\frac{1}{2}$ -day *post coitum* female embryos heterozygous for *Pgk-1*. Three of the eight were *Pgk-1^b/Pgk-1^a* heterozygotes, the other five were the reciprocal *Pgk-1^a/Pgk-1^b* heterozygotes (the first named *Pgk-1* allele is maternally inherited). The embryos of both crosses analysed were litter mates. Figure 2A, B shows representative gels of PGK-1 isozyme expression in tissues of *Pgk-1^a/Pgk-1^b* embryos.

Covariance among tissues

Table 1 shows the mean PGK-1A contribution in the six tissues from each female. As expected from earlier work (West, Frels, Chapman & Papaioannou, 1977; McMahon *et al.* 1981) only the maternally inherited *Pgk-1* allele is expressed in yolk-sac endoderm. In the remaining five tissues, both isozymes are expressed. The PGK-1A isozyme contributions are remarkably similar in all tissues from the same embryo and different from one sibling embryo to another. Table 2 gives the correlation coefficients between tissues: all pairs of tissues are found to be highly correlated. Moreover, when tested by a two-way analysis of variance (Appendix I), no systematic differences between tissues are detected in the PGK-1 isozyme contributions when the differences are averaged over embryos.

This finding is consistent with the view that all tissues are derived by random sampling of the same pool of cells after X-inactivation. Furthermore, this pool of cells is relatively small in size leading to considerable variation among embryos in PGK-1 isozyme contribution. Data from congenic C3H crosses differing at the PGK-1 locus (data not shown) show a similar wide variation. Therefore

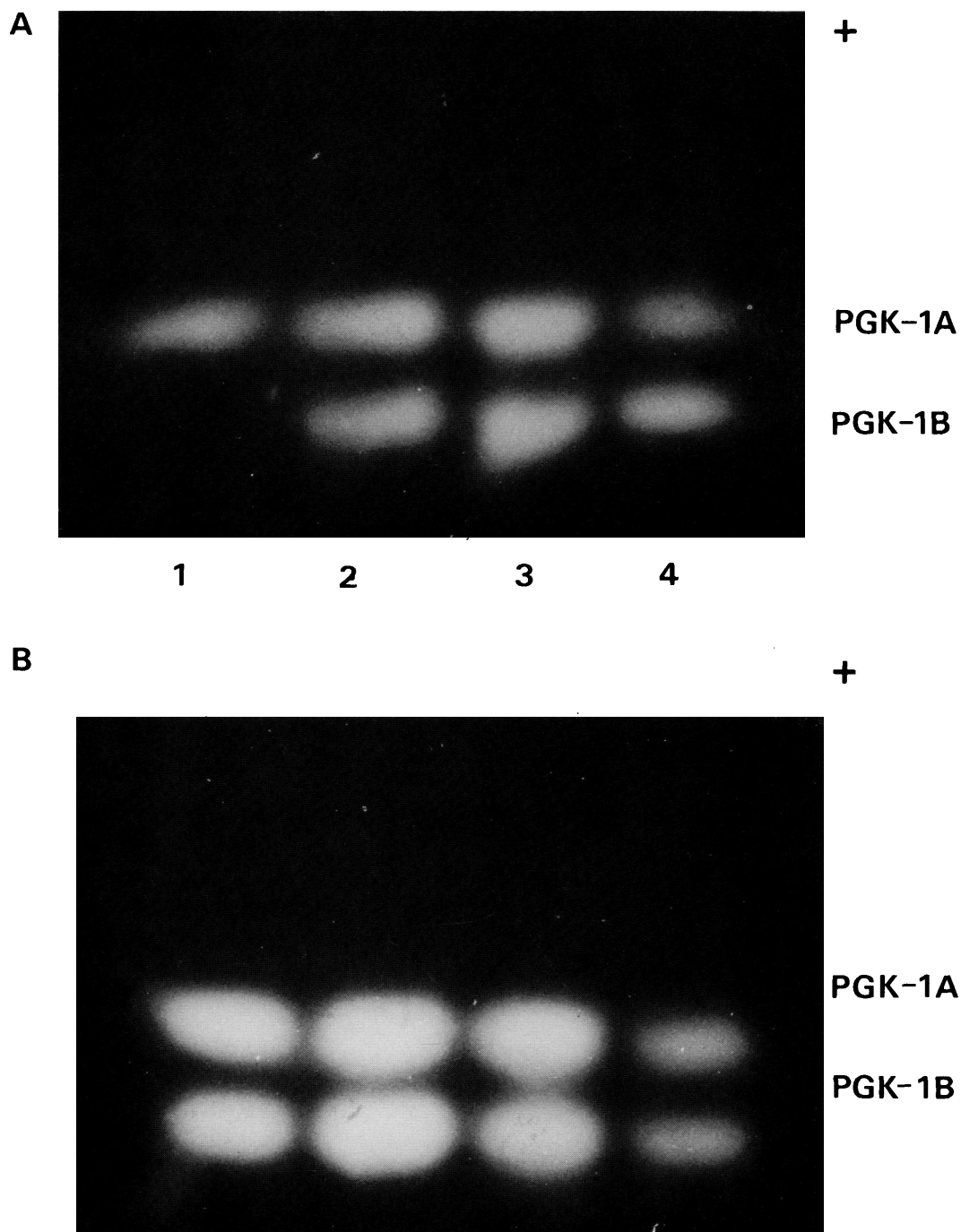


Fig. 2. PGK-1 isozyme expression in tissues from a *Pgk-1^a/Pgk-1^b* 12½-day *post coitum* female embryo. (A) PGK-1 expression in: 1, yolk-sac endoderm; 2, yolk-sac mesoderm; 3, neural ectoderm; 4, germ cells. (B) PGK-1 expression in: 1, yolk-sac mesoderm; 2, heart; 3, liver; 4, neural ectoderm.

Table 1. Mean percent PGK-1A expression in tissue samples from heterozygous 12½ day post coitum female embryos

Cross	Embryo	Yolk-sac endoderm	Mean percent PGK-1A isozyme activity*				Liver	Germ cells	Average
			Yolk-sac mesoderm	Neural ectoderm	Heart				
$Pgk\text{-}I^b/Pgk\text{-}I^b\bar{Q} \times Pgk\text{-}I^a/Y\bar{O}^{\bar{a}}$	1	0	58.7 ± 0.8	62.5 ± 1.5	62.1 ± 1.3	62.3 ± 1.1	59.3 ± 2.0	61.0	
	2	0	50.1 ± 1.5	50.2 ± 0.8	49.4 ± 0.4	49.0 ± 0.9	46.4 ± 1.0	49.0	
	3	0	57.5 ± 0.6	59.4 ± 1.5	56.3 ± 1.4	58.6 ± 0.9	63.3 ± 1.0	59.0	
$Pgk\text{-}I^a/Pgk\text{-}I^a\bar{Q} \times Pgk\text{-}I^b/Y\bar{O}^{\bar{a}}$	1	100	65.3 ± 1.5	56.8 ± 0.7	58.9 ± 0.5	57.8 ± 0.3	66.8 ± 0.5	61.2	
	2	100	72.3 ± 1.8	68.0 ± 3.5	70.1 ± 4.4	63.7 ± 2.3	66.9 ± 0.7	68.2	
	3	100	61.3 ± 1.0	52.1 ± 1.3	52.1 ± 0.7	50.6 ± 0.8	57.8 ± 1.4	54.8	
	4	100	51.1 ± 1.7	51.3 ± 0.5	48.6 ± 0.6	52.7 ± 0.5	46.1 ± 0.5	50.0	
	5	100	73.7 ± 1.3	68.8 ± 2.0	62.3 ± 0.6	63.9 ± 2.0	72.8 ± 0.5	68.3	
Average			61.3	58.6	57.5	57.3	59.9	58.9	

* PGK-1 isozyme expression in each heterozygous germ-layer tissue for each embryo was quantitated fluorometrically at a minimum of four time points in each of at least three separate independent electrophoretic runs of that tissue. The averages with standard errors of the independent runs are given. For the germ cells, averages with standard errors are calculated from multiple time-point quantitations for one electrophoretic run due to the more limited germ-cell tissue available per embryo.

* PGK-1 isozyme expression in each heterozygous germ-layer tissue for each embryo was quantitated fluorometrically at a minimum of four time points in each of at least three separate independent electrophoretic runs of that tissue. The averages with standard errors of the independent runs are given. For the germ cells, averages with standard errors are calculated from multiple time-point quantitations for one electrophoretic run due to the more limited germ-cell tissue available per embryo.

Table 2. *Correlation coefficients for percent PGK-1A isozyme expression between tissues from 12½-day post coitum heterozygous female foetuses*

	Yolk sac mesoderm				
Neural ectoderm	0.84**	Neural ectoderm			
Heart	0.83*	0.94***	Heart		
Liver	0.76*	0.94***	0.91**	Liver	
Germ cells	0.92**	0.82*	0.80*	0.81*	Germ cells

* = $P < 0.05$

** = $P < 0.01$

*** = $P < 0.001$

The data from Table 1 were tested for correlation of percent PGK-1A expression in all possible pairs of tissues analysed.

the above results reflect the limited size of the common pool of X-inactivated cells rather than the segregation of genes affecting the randomness of X-inactivation in the random-bred MF1 mouse stock.

Estimation of pool sizes

The theory behind the use of genetic mosaics to estimate precursor pool sizes assumes that, at the time immediately preceding the sampling event, cells are randomly distributed and the cells sampled are withdrawn from a pool at random. If the number of cells sampled for each tissue is relatively large, considerable cell mixing must occur if the tissues are still to reflect the initial composition of the common pool of X-inactivated cells following amplification. A further assumption, critical to these estimates is that there is no cell selection favouring one of the two distinct cell types arising after X-inactivation. The analysis of tissues of the foetal mouse ensures that the period over which cell selection might occur is relatively short.

From the covariance of tissue pairs from embryo to embryo and the independent variance of the tissues within embryos, it is possible to estimate the number, n_1 , of cells represented in the common pool from which the entire embryo is formed and the number, n_2 , of cells sampled at tissue foundation. A statistical approach similar to that of Nesbitt (1971) and described fully in Stone (1983), is applied in Appendix II. An estimate of 47 cells is obtained for n_1 and 193 cells for n_2 . The analysis of several tissues per embryo, and the multiple determinations required to achieve the accuracy of the PGK-1 quantitations, meant that a relatively small number of embryos could be analysed in this way. Therefore the estimate of n_1 has high intrinsic variability which is not easily quantifiable. The estimate of n_2 , an average value for all tissues, is relatively more precise (Appendix II).

These estimates were calculated assuming no experimental error in the replicate electrophoretic evaluations. When experimental error is taken into account, the estimates were unchanged as might be expected from the small standard errors in Table 1.

DISCUSSION

There is evidence that X-inactivation may occur at different times in different cell lineages as they differentiate in the early female mouse embryo (Monk & Harper, 1979; McMahon & Monk, 1982), and that preferential inactivation of the paternal X chromosome occurs in the early differentiating lineages, trophectoderm and primary endoderm (West *et al.* 1977; Takagi, Wake & Sasaki, 1978; Harper, Fosten & Monk, 1982; Papaioannou, West, Bücher & Linke, 1981). If non-random X-inactivation is produced by some imprinting of the paternal X chromosome which 'wears off' gradually, and if X-chromosome differentiation is linked to cell differentiation, then a sequence of differentiating embryonic lineages may show a progressive decrease in the proportion of cells with the maternal X chromosome active from 100 % towards 50 % (random inactivation); i.e. a temporal lineage map of the definitive germ layers could be constructed (Monk, 1978, 1981). From the results presented here, it is clear that X-inactivation is completed before the delineation of the yolk-sac mesoderm, the three germ layers and the germ line itself, and the question of whether these tissues 'bud off' in a sequence in time remains unresolved. As all tissues within an embryo show a similar proportion of cells with the *Pgk-1^a*-carrying X chromosome active, the mosaic composition among tissues reflects some common ancestral event confirming Nesbitt's (1971) earlier analysis using late replication of a translocated X chromosome as a cell marker.

The ancestral event which is common to all lineages may be: a) X-inactivation in a limited number of embryo precursor cells; b) the sampling of a number of cells from a population of X-inactivated cells, i.e. a subpopulation of cells which alone forms the entire embryo.

In both instances the results indicate that considerable growth and cell mixing must occur before sampling for the various lineages to explain the similarity in the mosaic composition of tissues within an embryo. It is not possible to distinguish between the alternatives above, though it is interesting to note that the second alternative implies redundancy in the epiblast cells in that some do not go on to form the embryo or epiblast-derived extraembryonic membranes.

Given these two alternatives, a statistical analysis can provide an estimate of the minimum number of cells present at X-inactivation. From the covariance of tissues, a value of 47 was estimated. The number of cells at the time of X-inactivation would be greater if a subpopulation of these cells is sampled as the embryo precursor cell pool.

As there are approximately 20–25 cells in the epiblast at 4½ days *post coitum*

(McLaren, 1976b; Gardner & Rossant, 1979) and 95–115 cells at $5\frac{1}{2}$ days *post coitum* (Snow, 1976), this places the earliest time of X-inactivation in the epiblast cells between $4\frac{1}{2}$ and $5\frac{1}{2}$ days *post coitum*. Rastan, Kaufman, Handyside & Lyon (1980) have demonstrated that at $5\frac{1}{2}$ days *post coitum*, the two X chromosomes in cells of the whole egg cylinder (epiblast and extraembryonic ectoderm and endoderm together) of female embryos stain differentially, and concluded that X-chromosome inactivation has occurred by this time. Studies on PGK-1 isozyme expression (McMahon & Monk, 1982) and dosage of HPRT (Monk & Harper, 1979) suggest X-chromosome inactivation in the epiblast occurs slightly later, although enzyme levels can only reflect genetic events which took place at some earlier time. All the evidence is in general agreement with X-inactivation in epiblast cells between $4\frac{1}{2}$ and 6 days *post coitum*.

Part of the variation among tissues in an embryo results from sampling events specific to that tissue. We assume that the mosaic composition of the representative tissues analysed for each germ layer reflects the mosaic composition of the germ layer itself. From the independent variance among tissues, an estimate of 193 (approximate 95 % confidence interval 120– ∞) cells was calculated for the primordial precursor pool size supposing this to be the same for each of the tissues analysed. As all pairs of tissues show similar high levels of correlations in the mosaic compositions measured, it is unlikely that any individual tissue is formed from a very much smaller number of cells than the mean value of 193 cells. If we assume a spatially contiguous sample of cells for a given tissue, this figure may well underestimate the actual number of cells if there is coherent clonal growth before tissue allocation (McLaren, 1972; Lewis, Summerbell & Wolpert, 1972; West, 1978).

Nesbitt (1971) calculated much smaller primordial precursor pool sizes (21–58) for several tissues (lung, abdominal fascia, spleen, thymus and melanocytes) than has been calculated here. The higher independent variance of tissues in Nesbitt's study may actually represent smaller primordial precursor pool sizes for the tissues examined or greater variability arising from the different experimental approach. The primordial pool size of human tissues from females heterozygous for electrophoretic variant forms of G6PD was calculated to be not less than 80 cells (Fialkow, 1973).

The primordial precursor pool size estimate of 193 for germ cells is of particular interest since previous workers have suggested the germ-cell line originates from few cells. Histochemical alkaline phosphatase staining of 8-day embryos (Mintz & Russell, 1957) indicates that the primordial germ-cell pool size may be as low as 10. However these cells may only represent the fraction of the total germ-cell population that has emerged from the alkaline-phosphatase-positive primitive streak; alternatively some germ cells may not stain positively, or are yet to acquire alkaline phosphatase activity. Observations on correlations of coat-colour and germ-line mosaicism resulting from radiation-induced or spontaneous mutation (Russell, 1964; Searle, 1978) also suggest low values of 5

to 10 original germ cells; Searle (1978) showed that although germ-line mosaicism was usually correlated with coat-colour mosaicism, there were a few animals that were non-mosaic in the germ line. We have no explanation for the discrepancy between these results and our observations on X-chromosome mosaicism for PGK-1 in the germ-line and somatic tissues. The possibility of somatic contamination of our germ-cell samples being responsible for the observed positive correlation seems unlikely since the germ cells isolated were at least 78 % pure (by alkaline phosphatase staining) when first isolated and further purified of contaminating blood cells before sampling for electrophoresis.

Although historically it has been long argued that the germ line originates from a small number of totipotent cells protected from differentiative events, the data presented in this paper suggest an alternative view. Rather than the germ line arising from a small number of cells set aside early on, it may be that it is derived from an appreciable number of cells remaining after all the somatic cell lineages of the foetus have been set aside; these remaining cells would then become subject to a process, possibly associated with meiosis, which restores totipotency.

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Appendix I

Analysis of variance for the two factors "embryo" and "tissue"

The following table gives an (unweighted) analysis of variance of the 40 PGK-1A percentages of Table 1, excluding the yolk-sac endoderm values.

Source	SS	df	Mean Square	F
Between embryos	1893.14	7	270.4	25 ($P < 0.001$)
Between tissues	89.21	4	22.3	2.0 ($P > 0.10$)
Residual	304.62	28	10.9	
Total SS	2286.97			

The non-significance of the differences between the tissues, averaged over embryos (which contrasts with the highly significant differences between embryos), shows that the data are consistent with the following additive model:

$$(\text{PGK-1 Percentage}) = \left(\begin{matrix} \text{Overall} \\ \text{Mean} \end{matrix} \right) + \left(\begin{matrix} \text{Embryo} \\ \text{deviation} \end{matrix} \right) + \left(\begin{matrix} \text{Random deviation for} \\ \text{each tissue within embryo} \end{matrix} \right)$$

Appendix II

Estimation of n_1 and n_2

n_1 is the number of X-inactivated embryo precursor cells and n_2 the number of cells sampled for each tissue considered. n_1 and n_2 may be estimated for the covariance within individuals and the independent variation of the different tissues, respectively; in the study of Nesbitt (1971), covariance between pairs of tissues was used to derive many independent estimates of n_1 . However, it is more appropriate to consider all tissues as "replicate" measurements and calculate a single estimate, \hat{n}_1 , of n_1 . Moreover, the data do not suggest that n_2 differs much from tissue to tissue, so we will calculate a single estimate, \hat{n}_2 , of n_2 for all tissues.

Let y_{ij} denote the average of the replicate session determinations of the PGK-1A percentage for tissue j taken from embryo i , $i = 1, \dots, 8$, $j = 1, \dots, 5$. Supposing any contribution of experimental assay error to y_{ij} is negligible, we have, following Nesbitt (1971),

$$\text{Var } \frac{y_{ij}}{100} = \left(\frac{1}{n_1} + \frac{1}{n_2} - \frac{1}{n_1 n_2} \right) pq$$

$$\text{Cov } \left(\frac{y_{ij}}{100}, \frac{y_{ij}}{100} \right) = \frac{pq}{n_1},$$

where p denotes the probability that X-inactivation will yield a PGK-1A cell type, estimated as 0.589 from Table 1, and $q = 1 - p$.

In our alternative analysis to Nesbitt's, these variances and covariances may be considered as the consequences of the additive model (as in Appendix I):

$$\frac{y_{ij}}{100} = p + e_i + t_{ij},$$

where e_i is the deviation for embryo i , with variance σ_e^2 , t_{ij} is the random deviation for tissue j from embryo i , with variance σ_t^2 , where the variances are given by,

$$\sigma_e^2 = \frac{pq}{n_1} \quad (1)$$

$$\sigma_t^2 = \frac{1}{n_2} \left(1 - \frac{1}{n_1} \right) pq \quad (2)$$

With the additional supposition that n_2 is the same for all tissues, estimates $\hat{\sigma}_e^2$ and $\hat{\sigma}_t^2$ may (following Snedecor & Cochran (1967), section 10.16): be calculated from the condensed analysis of variance table:

	SS	df	Mean Square
Between embryos	1893.14	7	270.4
Between tissues within embryos	393.83	32	12.3
Total	2286.97	39	

We get

$$\begin{aligned} \hat{\sigma}_t^2 &= 12.3 \times 10^{-4}, \quad \hat{\sigma}_e^2 = \frac{(270.4 - 12.3) \times 10^{-4}}{5} \\ &= 51.6 \times 10^{-4} \end{aligned}$$

Using equations (1) and (2), we get

$$\begin{aligned} \hat{n}_1 &= \frac{0.589 (1 - 0.589) \times 10^4}{51.6} \\ &= 47 \\ \hat{n}_2 &= \left(1 - \frac{1}{47} \right) \frac{0.589 \times (1 - 0.589) \times 10^4}{12.3} = 193 \end{aligned}$$

An approximate one-sided 95 % confidence interval for n_2 , based on the assumption that $\hat{\sigma}_1^2$ is proportional to a chi square with 32 degrees of freedom, gives the range $120 < n_2 < \infty$.